Neutral Sphingomyelinase

INSERT 1

The present invention relates to nucleic acids coding for eukaryotic neutral sphingomyelinase, and applications thereof.

Sphingomyelin is an essential component of plasma membranes. Degradation of sphingomyelin gives a number of substances having potential second messenger properties, e.g., ceramide, sphingosine, sphingosine-1-phosphate. Two sphingomyelin-cleaving enzymatic activities are known, namely that of lysosomal acid sphingomyelinase, and that of plasma-bound neutral sphingomyelinase.

Bacterial neutral sphingomyelinase is a secreted soluble protein.

The present invention for the first time provides nucleic acids coding for eukaryotic neutral sphingomyelinase. Eukaryotic neutral sphingomyelinase (nSMase) is characterized in that it cleaves sphingomyelin into ceramide and phosphocholine and that its activity depends on the addition of magnesium ions. It is a membrane-bound enzyme. Its maximum activity is achieved in the neutral pH range.

Figure 1 shows the gene sequence of human neutral sphingomyelinase.

Figure 2 shows the gene sequence of murine neutral sphingomyelinase.

Figure 3 shows the results of the Northern and Western blotting of nSMase-overexpressing cell lines.

Figure 4 shows the strategy for producing murine knockout mutants. The letters designate restriction sites.

Figure 5 shows constructs for obtaining transgenic mouse mutants.

Preferably, the nucleic acid according to the invention is a nucleic acid coding for the neutral sphingomyelinase of a mammal. More preferably, it codes for human or murine neutral sphingomyelinase. The corresponding nucleic acid sequences are disclosed as SEQ. ID. NO. 3 and SEQ ID. NO. 4, respectively.

Parts of the nucleic acid sequences are identical with the EST sequences AA028477 and AA013912 (murine) and W32352 and AA056024 (human).

When he knows the amino acid and nucleic acid structure of human and murine neutral sphingomyelinase, one skilled in the art can easily detect the corresponding nucleic acids and proteins from other eukaryotes, considering the high homology between human and murine nSMases. To do this, he can either use cross-reacting antibodies for a purification by specific affinity chromatography, or he can synthesize oligonucleotide primers on the basis of the nucleic acid sequence and amplify the desired nucleic acids in a cDNA library of the eukaryote using polymerase chain reaction. The corresponding cDNA library can be obtained in a per se known manner by isolating mRNA from a tissue sample, followed by reverse transcription. From the nucleic acid sequence, the amino acid sequence can be derived by means of the genetic code. Alternatively, it is also possible to search for homologous sequences in EST (expressed sequence tags) data bases and combine them.

The nucleic acids according to the invention are suitable for the expression of eukaryotic neutral sphingomyelinase in prokaryotic or eukaryotic systems. In addition, they are also suitable for expression of nSMase in vivo in a gene therapy, or especially, in the form of fragments with complementary structures, they are also suitable as antisense nucleotides for reducing the expression of nSMase.

The nucleic acids according to the invention can be prepared by chemical synthesis or by amplification in genetically engineered organisms by methods per se known to those skilled in the art.

The invention also relates to eukaryotic neutral sphingomyelinase obtainable by the expression of the nucleic acids according to the invention.

The nSMase according to the invention can be prepared by expression in genetically engineered organisms. Eukaryotic expression systems are particularly suitable. Appropriate eukaryotic expression systems are known to those skilled in the art, for example, pRc/CMV (Stratagene). Purification from genetically engineered organisms offers an easy and direct access to the nSMase according to the invention, especially in the case of overexpression, and in addition allows for the isolation thereof in larger quantities.

The eukaryotic neutral sphingomyelinase is preferably a mammal, especially human or murine, neutral sphingomyelinase. The amino acid sequences of the human and murine neutral sphingomyelinases are represented as SEQ. ID. NOS. 1 and 2.

The molecular weights of human and murine sphingomyelinases are 47.6 and 47.5 kDa, respectively. In contrast to bacterial nSMases, the mammal nSMases according to the invention do not contain a signal sequence at the N terminus. From the hydrophobicity analysis, it can be considered that two neighboring hydrophobic membrane domains at the C terminus are separated by eight amino acids. Therefore, the proteins appear to be integral membrane proteins whose catalytically active domain is directed towards the cytosol while only a small proportion of the enzymes contacts the extracellular environment. This is in contrast to bacterial nSMases which are secreted, soluble proteins, but in agreement with previous studies on the properties of neutral sphingomyelinases of mammals. According to a Northern blot analysis, the 1.7 kb mRNA of murine nSMase is expressed in all tissues. In the kidneys, brain, liver, heart and lungs, the Northern blot shows a strong signal while expression in the spleen appears to be low. This measurement was not in agreement with the measured enzymatic activities of the corresponding tissues. This speaks in favor of a post-transcriptional regulation of nSMase.

The pH optimum of the neutral sphingomyelinase according to the invention is within a range of from 6.5 to 7.5, with a K_m value for C18 sphingomyelin

within a range of from 1.0 to 1.5 x 10^{-5} M. The activity is dependent on the presence of magnesium ions; the addition of EDTA results in an inhibition of SMase activity, which can be restored, however, by the addition of Mn^{2+} or Mg^{2+} ions. The addition of 0.3 to 0.5% Triton X-100 increases the enzymatic activity. The activity is not affected by a treatment with DTT or 2-mercaptoethanol whereas the addition of 20 mM glutathione led to inhibition. The activity of nSMase is not restricted to sphingomyelin; the structurally related phosphatidylcholine was also cleaved with about 3% activity.

Also claimed are variants of the eukaryotic neutral sphingomyelinase. The term "variants" encompasses both naturally occurring allelic variations of the eukaryotic neutral sphingomyelinase and proteins prepared by recombinant DNA technology (especially by in-vitro mutagenesis using chemically synthesized oligonucleotides) followed by expression which correspond to eukaryotic neutral sphingomyelinase in terms of biological and/or immunological activity. This may include the deletion, insertion or conservative substitution of amino acids. "Conservative substitution" means that an amino acid is substituted by another amino acid having similar physicochemical properties.

Thus, for example, the following amino acids are interchangeable: serine and alanine; alanine and glycine; methionine and serine; lysine and arginine; lysine and serine.

In particular, the term "variants" also includes N-terminally and/or C-terminally truncated proteins as well as acetylated, glycosylated, amidated and/or phosphorylated derivatives.

At least part of the activity of nSMase seems to reside in the C-terminal region since the fragment 1-282 of murine nSMase failed to exhibit an increase of sphingomyelinase activity when expressed in HEK293 cells. This invention also relates to C-terminal fragments of nSMase. Compounds in which nSMase or its variants are coupled with other molecules, such as dyes, radionuclides or affinity components, are also variants according to the invention.

Also claimed are nucleic acids coding for eukaryotic neutral sphingomyelinase or being complementary to such nucleic acids. The nucleic acids may be, for example, DNA, RNA, PNA or nuclease-resistant analogues thereof. In particular, nuclease-resistant analogues include those compounds which have the phosphodiester linkage modified by hydrolysis-stable compounds, such as phosphothioates, methylphosphonates or the like.

Especially short fragments of the nucleic acids are suitable as antisense nucleotides. For reasons of specificity, they should preferably contain more than 6, more preferably more than 8 and most preferably more than 12 nucleotides. For reasons of diffusion and costs, they usually have a length of less than 30 nucleotides, preferably 24 or less, and more preferably 18 or less nucleotides.

The invention also relates to derivatives of nucleic acids which are coupled to other molecules for diagnostic or therapeutic purposes, for example, to fluorescent dyes, radioactive labels or affinity components, and fragments of the nucleic acids according to the invention, and the nucleic acids complementary to these nucleic acids, and variants of the nucleic acids.

"Fragments" as used herein means nucleic acids truncated at the 5' or 3' or at both ends. The term "variants" means that these nucleic acids will hybridize with the nucleic acid according to the invention or with nucleic acids complementary thereto under stringent conditions. The term "stringent conditions" means that the hybridization is performed under conditions in which the temperature is even lower by up to 10 °C than the temperature (conditions being otherwise identical) just low enough for exactly complementary nucleic acids to anneal. For example, if an exactly complementary nucleic acid will anneal down to a temperature of about 55 °C under given conditions, then stringent conditions are temperatures of equal to or higher than 45 °C. Preferably, the temperature range for stringent conditions is within 5 °C, more preferably within 3 °C.

Further, the invention relates to antibodies directed against the nSMase according to the invention or the nucleic acids according to the invention.

These substances are suitable, in particular, for use in diagnostics, in immuno-assays per se known to those skilled in the art, for histological studies and as medicaments for the treatment of conditions associated with an overexpression of nSMase. Such antibodies according to the invention can be obtained by methods per se known to those skilled in the art through immunization with nSMase, nucleic acids according to the invention or peptide and nucleic acid fragments in the presence of adjuvants.

Further, the invention relates to cell lines which overexpress the nSMase according to the invention. Such cell lines can be obtained by transfection with vectors containing the nucleic acids according to the invention coding for nSMase. In the case of eukaryotic cell lines, for example, transfection may be effected by electroporation. Preferably, the cell lines are stably transfected.

In this connection, "overexpression" means that the cell line has a higher activity of nSMase than cell lines which have not been transfected with the nucleic acids according to the invention. For example, suitable eukaryotic cell lines include the cell lines U937, HEK 293 or Jurkat.

In experiments, the cell lines exhibited a specific nSMase activity of between 0.3 and 10 µmol/mg of protein/hour.

Figure 3 shows the Northern and Western blot analysis of nSMase expression in transfected cell lines. Portion A shows the result of a RT PCR of the whole cell RNA with primers hybridizing with human and murine nSMase cDNAs. Portion B shows the T PCR of the whole RNA with primers hybridizing with human β-actin cDNA as a control. Portion C shows the Western blot of the plasma membrane protein extract of different HEK 293 cell lines after SDS polyacrylamide gel electrophoresis and hybridization with polyclonal anti-nSMase antibodies.

The addition of 0.5 mM arachidonic acid resulted in a threefold increase of nSMase activity in the overexpressing HEK cells.

The invention further relates to a transgenic mammal which exhibits an overexpression (gain of function) or a genetic deficiency or defect (loss of function) for the nSMase according to the invention. The mammal is preferably a rodent, especially a mouse. Such transgenic mammals can be obtained by methods per se known to those skilled in the art and are especially suitable for elucidating the function of neutral sphingomyelinase. For transgenic mammals, defined gene constructs are injected into the pronucleus of a fertilized egg cell by DNA microinjection to achieve the expression of an additional gene. By selectively changing a gene in the genome of ES cells which are subsequently injected in blastocysts, the function of a gene is switched off.

The strategy and constructs for generating the mouse mutants are shown in Figures 4 and 5.

The transgenic animals are preferably animals in which the gene can be switched on and off temporally and in a tissue-specific way by external induction. Such transgenic mammals are especially suitable for elucidating the metabolic and signal transduction pathways related to the nSMase according to the invention; this in turn enables diagnostic or therapeutic applications. In particular, the transgenic mammals are suitable for the screening of pharmaceutically active substances.

The eukaryotic neutral sphingomyelinase according to the invention, the nucleic acids according to the invention and the antibodies according to the invention can be contained in medicaments and diagnostic agents, optionally together with further auxiliary agents. Such medicaments and diagnostic agents are suitable for the diagnosis and treatment of diseases based on over- or underexpression and/or an increased or reduced activity of eukaryotic neutral sphingomyelinase and/or disorders of cell proliferation, cell differentiation and/or apoptosis.

In particular, these are diseases in which inflammation processes, cell growth disorders and metabolic disorders are involved. For example, they may be cancers or disorders of cholesterol homeostasis (atherosclerosis).

A pharmaceutical screening method according to the invention relies on a change of the expression or activity of the nSMase according to the invention in nSMase-overexpressing cell lines upon the addition of at least one potential pharmaceutically active substance. Thus, the cell lines are suitable, in particular, for developing and testing pharmaceutical leading structures.

The invention will be further illustrated by the following Examples.

Example 1

Cloning of the nucleic acid

The inventive nucleic acids coding for neutral sphingomyelinase were cloned into the NotI restriction sites of the cloning site of the eukaryotic expression vector pRc/CMV (Stratagene). The sequences of the resulting DNAs were obtained by sequencing using a Perkin-Elmer DNA sequencer 377A.

Example 2

Cloning of the RNA

The whole RNA was isolated from different organs of eight three-week-old CD1 mice according to known methods, and poly(A^+) RNA was isolated by affinity purification on oligo(dT) cellulose (Boehringer Mannheim, Germany) according to standard methods.

Example 3

Overexpressing cell lines

U937 cells were grown in PRMI 1640 medium with 10% fetal calf serum, 1 µg/ml penicillin/streptomycin and 0.03% glutamine at 37 °C and 5% CO_2. By electroporation with a Gene Pulser (Bio-Rad), 5 x 10^6 cells were transfected with 1 µg of linearized plasmid DNA coding for the nSMase according

to the invention. The selection of stable clones was effected by using 1 mg/ml geneticin (G418, Life Technologies, Gaithersburg, MD).

The nSMase purified from the cell lines exhibited a specific activity of between 0.3 and 10 µmol/mg of protein/hour. Its pH optimum was at 6.5 and 7.5. The K_M value for C18 sphingomyelin was from 1.0 to 1.5 x 10^{-5} M. The activity was dependent on the presence of magnesium ions; the addition of EDTA inhibited the activity.

Example 4

Measurement of nSMase activity

The enzymatic activity was examined in cells and murine tissues. The cells were washed twice with ice-cold PBS and sedimented at 1,000 x g. The pellet was resuspended in lysis buffer, and the cells were disrupted by repeated cycles of freezing and thawing. After centrifugation at 2,500 x g for 2 min, extraction with lysis buffer containing 0.2% Triton X-100 was performed, followed by centrifugation at 100,000 x g for 15 min.

Tissue from three-week-old mice was homogenized in cold lysis buffer. The quantity of protein or homogenized tissue to be examined was incubated with 10 nM (80,000 dpm) [N-$^{14}CH_3$]sphingomyelin for 30 min at 37 °C in a total volume of 200 µl. Then, 100 µl of water was added, and unreacted substrate was removed by extraction with chloroform-methanol (2:1, v/v). The radioactivity of the aqueous phase containing the enzymatically released phosphocholine was measured in a scintillation counter.

Example 5

Polyclonal antibodies

Rabbits were immunized with the synthetic peptide CDPHSDKPFSDHE (corresponding to amino acids 261 through 273 of murine nSMase), coupled to keyhole limpet hemocyanin. The polyclonal antibody serum was purified by

chromatography on hydroxyapatite and affinity chromatography on a column having the above mentioned synthetic peptide bound thereto.